HAYATI Journal of Biosciences 22 (2015) 186–190

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Contents lists available at ScienceDirect

HAYATI Journal of Biosciences

journal homepage: http://www.journals.elsevier.com/hayati-journal-of-biosciences

Original research article

Antibacterial, Antifungal and Anticancer Activity of Five Strains of Soil Microorganisms Isolated From Tangkuban Perahu Mountain by Fermentation

Desak Gede Sri Andayani,[1*] Ukan Sukandar,[3] Elin Y. Sukandar,[2] I. Ketut Adnyana[2]

[1] *Research Center for Chemistry, Indonesian Institute of Sciences, Bandung, Indonesia.*
[2] *The School of Pharmacy, Institut Teknologi Bandung, Bandung, Indonesia.*
[3] *Chemical Engineering, Institut Teknologi Bandung, Bandung, Indonesia.*

ARTICLE INFO

Article history:
Received 1 August 2015
Accepted 24 December 2015
Available online 5 February 2016

KEYWORDS:
antibacterial, antifungal and anticancer activities,
ethyl acetate extraction,
fermentation,
identification

ABSTRACT

Microorganisms were isolated from soil taken from Tangkuban Perahu mountain. Five strains were investigated in this study, designated TP1, TP2, TP3, TP4, and TP5, respectively. Morphological, biochemical and molecular identifications were conducted for all five strains. These isolates were shown to be closely related to *Nocardia sp*. YIM 65630 (90%), *Streptomyces galbus* (99%), *Aspergillus unguis* (86%), *Paecilomyces marquandii* (100%) and *Nocardia niigatensis* (95%), respectively. Production of antibacterial, antifungal and anticancer metabolites was done by fermentation. Screening for bioactivity of five isolates was done by testing the fermentation broth against resistant and pathogenic bacteria, fungi and T47D breast cancer cell line. TP2 strain showed the best bioactivity; the metabolite was purified by extraction with ethyl acetate. Antibacterial, antifungal and anticancer activities from the ethyl acetate extract of TP2 strain were tested by agar diffusion, microdilution and MTT. The extract was shown to be active against methicillin resistant *Staphylococcus*, methicillin sensitive *Staphylococcus aureus*, methicillin resistant coagulase negative *Staphylococcus*, vancomycin resistant *Enterococcus*, *Escherichia coli*, *Microsforum gypseum* with the minimum inhibitory concentration (μg/mL) and diameter of inhibition (mm): 150, 35; 150, 30; 300, 35; 300, 35; 300, 29; 4.7, 36, respectively. The IC_{50} value of the T47D cell line was 457 μg/mL.

1. Introduction

Soil microorganisms from the group of actinomycetes are well accepted to be the best producer of antibiotics. The study of the actinomycetes group through isolation, identification, and screening for potential bioactivity is expected to provide further knowledge about the diversity of the genera and species and to render the potential to find new antibiotics. Volcanic soil that is rich in sulfur is a potential source of medicinal raw materials that come from nature. Actinomycetes and fungi that are found around such an extreme area have adapted to utilize sulfur as nutrition, while at the same time utilizing this as a defense against other microorganisms. Fermentation technology can be used to produce such secondary metabolites of microorganisms to increase the metabolite product for exploitation by the pharmaceutical industry. To date, antibiotics have been used as a treatment against infection diseases that are caused by resistant and pathogenic bacteria, fungi and viruses. The isolation of new microorganisms and their new metabolites remains sought after because antibiotic resistance is becoming significance. Attempts have been made by various researchers to achieve these objectives. Antibiotics are secondary metabolites that are synthesized by a specific microorganism, while it is not necessary for that organism to live and grow. Various secondary metabolites are produced by actinomycetes and fungi groups around volcanic soil. Further study of those microorganisms can uncover the potential of such products to be exploited for the benefit of humankind (Berdi 2005; Boca Raton 2005). The aim of this study was to screen the highest antimicrobial and anticancer activity of strains isolated from Tangkuban Perahu mountain. Of the five investigated strains, TP2 showed the highest antimicrobial and anticancer activities against resistant and pathogenic bacteria, fungi, and breast cancer cell line cells.

* Corresponding author.
E-mail address: desakgedesa@gmail.com (D.G.S. Andayani).
Peer review under responsibility of Institut Pertanian Bogor.

http://dx.doi.org/10.1016/j.hjb.2016.01.003

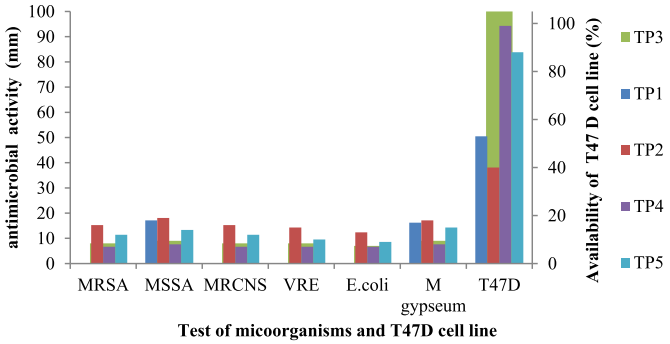

Figure 1. Screening of bioactivity of fermentation broth of TP1, TP2, TP3, TP4, and TP5 strains.

2. Material and Methods

2.1. Isolation of actinomycetes and fungi

One gram of dried soil sample was taken in 100 mL of sterile distilled water and mixed thoroughly in a shaker for 15 minutes at 120 rpm. One mL of different aqueous dilutions (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}, 10^{-7} and 10^{-8}) of the suspension was applied onto plates and 16 mL of melted potato dextrose agar (PDA) medium at around 40 °C was added to it. After gently rotating, the plates were incubated at 28 °C for 7–14 days. Selected colonies (rough, chalky) of actinomycetes and fungi were transferred from the mixed culture of the plates to respective agar plates and incubated at 28 °C for 7 days. The plates containing pure cultures were transferred and stored at 4 °C in the shape of slant agar (Demain and Solomon 1986).

2.2. Taxonomical characterization

The cultural characteristics of pure isolates in various media were recorded after incubation for 7–14 days at 28 °C. The active purified isolates of actinomycetes and fungi were identified up to the species level by comparing their morphology of spore bearing hyphae with an entire spore chain and structure of spore chain with the actinomycetes and fungi morphologies as described by *Bergey's Manual of Systematic Bacteriology*. The gram-staining method was applied to pure colonies. Briefly, a smear of culture was put on a clean glass slide and heated gently over a flame. The smear was covered with a thin film of crystal violet for 1 minute and washed gently in slow running tap water. Gram's iodine solution was flooded over the smear for 1 minute and washed with tap water. Alcohol was used to decolorize the smear until the violet color ceased to flow away. The slide was then washed with water and counter stain safranin was flooded over the smear for 2 minutes, after which the slide was washed, drained, air-dried, and viewed under a microscope. A culture retaining the violet color indicated that it is a gram-positive organism. (Holt *et al.* 1994; Cappuccino and Sherman 2005).

Molecular identification of TP2 strain was carried out based on a partial analysis of the 16S ribosomal RNA of the bacteria. Isolation of DNA was carried out by inoculating the TP2 strain in medium Yeast Starch Agar, and next incubating it for 48 hours. Mycelia biomass was then harvested for a DNA extraction process. Extraction of the genomic DNA of TP2 strain as a template for a polymerase chain reaction (PCR) was carried out using the GES method (Pitcher *et al.* 1989), after which the 16S rRNA gene was amplified by PCR. The primary amplification by PCR of 16S rRNA used 9F (5′GAGTTTGATCCTGGCTCAG–3′) and 1541 R (5-AAGGAGGTGATCCAACC-3′) (Hiraishi *et al.* 1995). PCR purification (LaMontagne *et al.* 2002) and continued through a sequencing cycle. The purification of this product was repeated by ethanol purification. The analysis of the nitrogen base sequence was read using an automated DNA sequencer (ABI PRISM 3130 Genetic Analyzer) (Applied Biosystems).

2.3. Sequence analysis

The raw data from the sequencing were then trimmed by the MEGA 4 program and assembled by the BioEdit program, and finally converted to the FASTA format. The DNA sequencing data in FASTA format were analyzed using the BLAST program to identify the homology using the online resources of National Center for Biotechnology Information (www.ncbi.nlm.nih.gov) and DNA Data Bank of Japan (www.ddbj.nig.ac.jp). The last step of identification was analysis of the phylogenetic tree using Clustal X program (Thompson *et al.* 1997) and neighbors-joining (NJ) plot program (Felsenstein 1985; Saitou and Nei 1987).

2.4. Antibacterial, antifungal, and anticancer screening by fermentation

The TP1, TP2, TP3, TP4, and TP5 strains were inoculated into fermentation medium, which consisted of soluble starch 1%, glucose 1%, yeast extract 0.5%, tryptone 0.25% and $CaCO_3$ 0.1%. This will be referred to as Medium A. Fifty milliliters of Medium A was prepared in a 250 mL capacity Erlenmeyer flask and then sterilized. Ten percent of starting inoculum (5 day old culture) was introduced into the sterile broth. The flask was incubated at 28 °C at 180 rpm for 48 days. After the incubation period, the broth was separated by centrifugation at 4500 rpm for 15 minutes. Cell-free supernatant was applied to the bacterial and fungal test microbes. The growth inhibition was determined by agar diffusion and the number of living T47D cell line cells (%) was determined by the formula (sample-blank)/(T47D cell line − blank) × 100%. An isolate with a low percentage of living T47D cell line cells indicates that it has a high anticancer potential (Mosmann 1983).

2.5. Fermentation condition and extraction of antibacterial, antifungal and anticancer metabolites

Based on the aforementioned results, the fermentation conditions were optimized for TP2 strain. Five liters of Medium A were prepared in a 10 L capacity fermenter and then sterilized. Ten

Table 1. Morphology of strains of TP1, TP2, TP3, TP4 and TP5

No	Strains	Macroscopic colony and microscopic cell characteristics
1	TP1	Circular (round), entire, umbonate, moderate colonies, chalk and rough, pigmented, white, dark brown-black reverse, slow growth, the color of young culture is white and the old culture is white/light-grey. Spore chains are spiral, gram positive.
2	TP2	Circular (round), entire, umbonate, pigmented, heavy grey, light-brown reverse. Cell with long hypha and spore chain with spiral filament. Gram positive, aerial mycelium.
3	TP3	The colony growth quickly to green color and white at the edge. Young culture was soft and velvety. Heavy brown to red in reverse. Septa hypha, no cleistothecia, sclerotia and coremia, straight conidiophore. Branch shape at the end of conidiophore and metulae. In metulae reside sterigmata. At the end of sterigmata conidia adhere making a conidia chain, subglobose conidia and no chlamydospora.
4	TP4	A light white color, velvety and hairy, septa hypha. Brown reverse, no cleistothecia, sclerotia and coremia. Straight conidiophore. Branch-shape hypha.
5	TP5	Circular, entire, umbonate, pigmented, pale white, brown reverse, cell with short hypha and chain with spiral filament. Gram positive. Aerial mycelium.

Table 2. Physiological and biochemical properties of TP1, TP2 and TP5 strains

Strain properties	TP1	TP2	TP5
Motility	Motile	Motile	Motile
Biochemistry assay			
Starch hydrolysis	Positive	Positive	Positive
Fat hydrolysis	Negative	Negative	Negative
Casein hydrolysis	Positive	Positive	Positive
Liquefaction of gelatin	Negative	Negative	Negative
Utilize of carbon source			
Inositol	Negative	Negative	Positive
Glucose	Positive	Positive	Positive
Mannose	Negative	Negative	Positive
Sorbitol	Positive	Negative	Positive
Fructose	Negative	Negative	Positive
Lactose	Negative	Negative	Positive
Maltose	Negative	Negative	Positive
Galactose	Negative	Negative	Positive
Xylose	Positive	Positive	Positive
Sucrose	Negative	Negative	Positive
Arabinose	Positive	Positive	Positive
Growth in NaCl 10%	Negative	Negative	Negative
Production of H_2S	Negative	Negative	Negative
Production of indole	Negative	Negative	Negative
Degradation of urea	Negative	Negative	Positive
Citrate reduction	Negative	Negative	Positive
Methyl red	Negative	Negative	Negative
Voges Proskauer	Negative	Negative	Negative
Simmon's citrate	Negative	Negative	Negative
Growth in 1% of Tryptone broth	Pellicle	Pellicle	Pellicle

percent of starter inoculum (5 day old culture) was added to 50 mL, then to 500 mL and the remainder to the 5 L of Medium A. The culture was incubated at 28 °C at 180 rpm at pH 7 for 48 hours. After the incubation period, the broth was separated by centrifugation at 4500 rpm for 15 minutes. Cell-free supernatant of TP2 strain was extracted with ethyl acetate. The filtrate and solvent were mixed at a 1:1 (v/v) ratio and the mixture was transferred to a separating funnel and shaken vigorously. Extraction was continued up to three times with the same solvent. The organic layer was collected and solvent was evaporated using a vacuum rotary evaporator at a temperature of 40 °C (Michael and Fikret 2002; Satyajit *et al.* 2006). Ethyl acetate extract free from organic solvent was tested with regards to its activities against test microbes and T47D cell line. Growth inhibition was determined by agar diffusion, the minimum inhibitory concentration (MIC) (µg/mL) was determined by microdilution, and the IC_{50} value (µg/mL) of the available (%) T47D cells was determined with the MTT method (Mosmann 1983; Freimoser *et al.* 1999; Michael and Fikret 2002).

2.6. Test organisms

The following test organisms were used to test the MIC and diameter of inhibition (mm) of the ethyl acetate extract: methicillin resistant *Staphylococcus aureus* (MRSA), methicillin sensitive *Staphylococcus aureus* (MSSA), methicillin resistant coagulase negative *Staphylococcus* (MRCNS), vancomycin resistant *Enterococcus* (VRE), *Escherichia coli*, *Microsforum gypseum* and T47D breast cancer cell line.

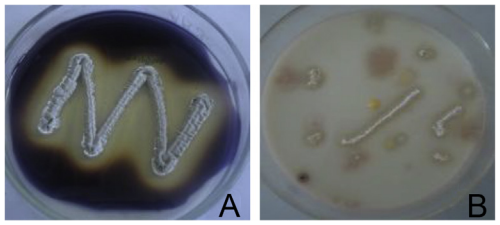

Figure 3. Production of (A) amylase and (B) protease by TP2 strain.

3. Results

3.1. Isolation of actinomycetes and fungi and screening of bioactivity

Three active strains of actinomycetes and two active strains of fungi were recovered from volcanic soil samples collected from Tangkuban Perahu mountain (West Java Province, Indonesia) using PDA. The isolated cultures are designated TP1, TP2, TP3, TP4, and TP5. The fermentation broth of the five strains were screened against resistant and pathogenic bacteria, fungi, and T47D cell line. The result of the bioactive screening was that all isolates showed antimicrobial and anticancer activities, with the range of diameter of inhibition being: 7–16 mm against MRSA, 8–19 mm against MSSA, 7–16 mm against MRCNS, 7–15 mm against VRE, 7–13 mm against *E. coli*, 8–18 mm against *M. gypseum* and 40%–100% T47D cells available; the highest bioactivity was shown by TP2 strain against MRSA (16 mm), MSSA (19 mm), MRCNS (16 mm), VRE (15 mm), *E. coli* (13 mm), *M. gypseum* (18 mm) and 40%–100% available T47D cells (Figure 1), and therefore TP2 was chosen for further study as a producer strain.

3.2. Morphological, physiological, biochemical, and molecular characteristics of strains

The study of characteristics of strains covers morphological, physiological, biochemical, and molecular aspects rendered in Tables 1 and 2, Figures 2–4.

The colors of the substrates mycelium and aerial spore mass were varied. All strains produced diffusible pigments on PDA media. Aerial hyphae of strain TP2 was differentiated into long spiral chains of cylindrical spores but strains TP1 and TP5 had a short chains. The strain TP2 produced long chain rectiflexible spores. On the other hand, the vegetative hyphae of all isolate branched but not fragmented were detected (Figure 1 and Table 1). The utilization and hydrolysis of carbohydrate, nitrogen, protein, and other characteristics are summarized in Table 2. Morphological examination (Figure 2, and Table 1) and further comparison of physiological and biochemical characteristics among the strains (Table 2) of TP1–TP5 suggest that TP1 and TP5 belong to *Nocardia* genera, TP2 belongs to *Streptomyces* genera, TP3 belongs to *Aspergillus* genera and TP4 belongs to *Paecilomyces* genera.

Analysis of the 16S rRNA gene for actinomycetes and 18S rRNA or ITS gene for fungi is an important tool for correct identification of

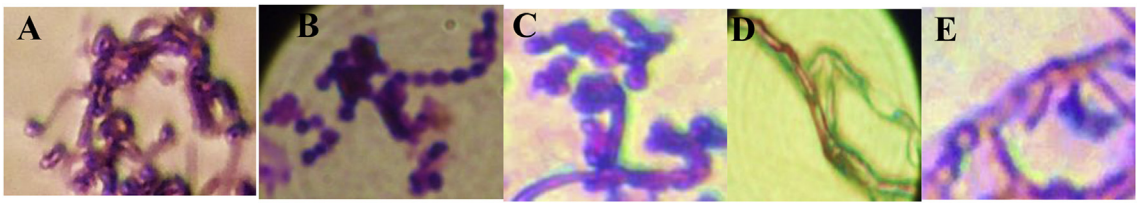

Figure 2. Hypha and chain of spora of (A) TP1, (B) TP2, (C) TP3, (D) TP4, and (E) TP5 strains in potato dextrose agar medium viewed by light microscope (100×).

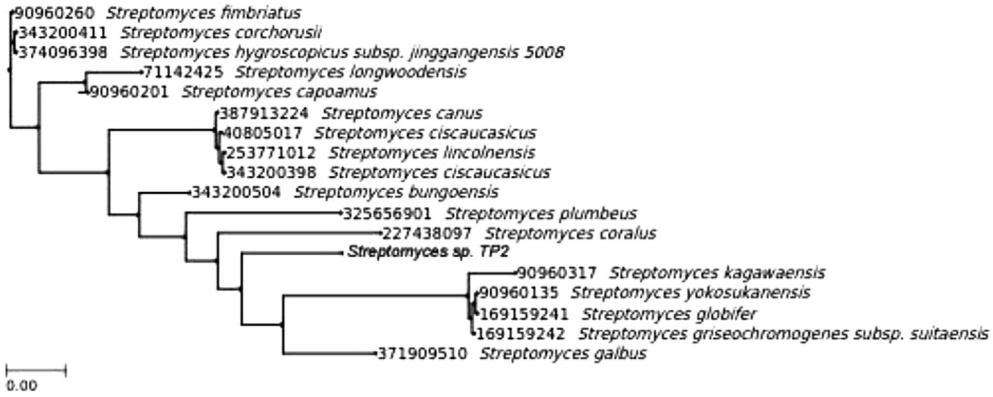

Figure 4. Phylogeny tree of TP2 strain.

Table 3. Result of molecular identification of 16S rRNA of strains

No	Strains	Homology (%)	Accession number
1	*Nocardia sp.* YIM 65630 TP1 strain	90	GU367167.1
2	*Streptomyces galbus* TP2 strain	99	NR_026178.1
3	*Aspergillus unguis* TP3 strain	88	FJ878626
4	*Paecilomyces marquandii* TP4 strain	100	FJ765026
5	*Nocardia niigatensis* TP5 strain	95	NR_043916.1

microbial species. The BLAST analysis of 16S rRNA gene sequences of strains of TP1, TP2 and TP5, and 18S rRNA or ITS of gene sequences of strains of TP3 and TP4 using the National Center for Biotechnology Information and DNA Data Bank of Japan databases shows that strains of TP1, TP2, TP3, TP4 and TP5 have the highest similarity with *Nocardia sp.* YIM 65630 (90%), *Streptomyces galbus* (99%), *Aspergillus unguis* (86%), *Paecilomyces marquandii* (100%), and *Nocardia niigatensis* (95%) respectively (Table 3).

The family and phylogenetic trees of TP2 strain were analyzed using the Clustal X program and the NJ plot program. To construct the phylogenetic trees, the homology sequence from the BLAST and FASTA format results was calculated for the data of three constructions by Clustal X, and then the calculated data were converted into trees with NJ plot. The NJ method is a simple program to construct the phylogenetic tree from evolutionary distance data by finding the pairs of operational taxonomic units (neighbors) that minimize the total branch length at each stage of clustering of neighbors starting with a star-like tree. The phylogenetic trees of 16S rRNA are shown in Figure 4.

3.3. Fermentation process and extraction of strain TP2

During the production of secondary metabolites, monitoring was done to the growth of microbes and measurement was undertaken of the use of sources of carbohydrate and nitrogen. In Figure 4, it can be seen that microbes used glucose as a source of carbon for their growth and formation of antibiotics. This was continued with the use of starch when the glucose ran out. This occurs because glucose has a simple sugar structure so that it can easily be degraded by microbes. The source of nitrogen was degraded last by the microbes for their growth and formation of secondary metabolites. Beginning the 20th hour through the 46th hour, a development of microbes in a stagnant fashion was observed, during which the use of sources of carbon and nitrogen remained constant. Under these circumstances, the microbes entered a phase known as the stationary phase, during which antibiotics are usually formed. The harvesting of secondary metabolites produced by TP2 strain was done at the hour with the highest development, namely the 46th hour, because at the 47th hour the growth slowly decreased, approaching the death phase. At the hour with the highest development, the liquid of fermentation was extracted with the organic solvent ethyl acetate. The extract of ethyl acetate was tested against test microbes and T47D cell line. The diameter of inhibition (mm) and the MIC value (μg/mL) of the ethyl acetate extract of TP2 strain were as follows: MRSA (35; 150), MSSA (30; 150), MRCNS (35; 300), VRE (35; 300), *E. coli* (29; 300), fungi, *M. gypseum* (36; 4.7) and the IC_{50} value of anticancer activity was 457 μg/mL (Table 4, Figure 5).

4. Discussion

From the volcanic soil samples collected from Tangkuban Perahu mountain, three actinomycetes and two fungi were isolated. All of the isolates demonstrated antibiotic biological activities, such as broad-spectrum and narrow-spectrum. The highest activity of five strains against resistant and pathogenic bacteria, and fungi was shown by TP2 strain. Figure 1 shows that the fermentation broth of TP2 strain had the lowest T47D cell availability (40%) of five isolates, which suggests that TP2 strain has the highest anticancer potential (Sugiyanto *et al.* 2003).

Table 4. Diameter of inhibition (mm), MIC, and IC_{50} (μg/mL) of ethyl acetate extract of TP2 strain

No	Test of organisms	Inhibition of diameter (mm)	MIC (μg/mL)	IC_{50} (μg/mL)
1	MRSA	35	150	–
2	MSSA	30	150	–
3	MRCNS	35	300	–
4	VRE	35	300	–
5	*Escherichia coli*	29	300	–
6	*Microsforum gypseum*	36	4.7	–
7	T47D cell line	–	–	457

MIC = minimum inhibitory concentration; MRSA = methicillin resistant *Staphylococcus aureus*; MSSA = methicillin sensitive *Staphylococcus aureus*; VRE = *vancomycin resistant Enterococcus*.

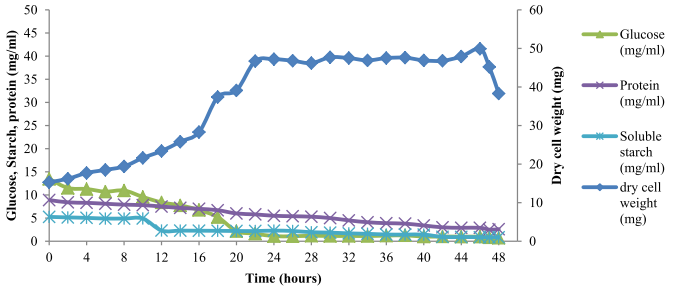

Figure 5. Characterization of strain TP2 in fermentation process for 48 hours, 28 °C, pH 7, 1 vvm, and 180 rpm.

DNA sequencing data of five strains was continued in FASTA format using the BLAST program. 16S rRNA or 16S ribosomal RNA is a component of the 30S small subunit of prokaryotic ribosomes. It is 1542 kb (or 1542 nucleotides) in length. The gene coding for it is referred to as 16S rRNA and is used for reconstructing phylogenies (Felsenstein 1985; Saitou and Nei 1987; Hiraishi *et al*. 1995). The results of the homology analysis of the ribosomal DNA of the five strains using 16S rRNA are shown on Table 3.

The search for novel metabolites especially from actinomycetes and fungi requires a large number of isolates to discover a novel compound of pharmaceutical interest. The search will be more promising if diverse actinomycetes and fungi are sampled and screened. For this reason we focused on actinomycetes and fungi from volcanic soil expected to produce different types of secondary metabolites and some of these chemical compounds are toxic to soil microorganisms including actinomycetes and fungi. However, adaptation has led in turn to the actinomycetes and fungi to produce their own secondary metabolites (Omura 1992).

The production of metabolite by fermentation process of TP2 strain was done for 46 hours. Metabolites with antibacterial, antifungal, and anticancer activities were induced by the decrease of glucose, protein and starch concentrate in the growth medium. During that time the microorganism used the carbon and nitrogen source for making antibiotics.

The ethyl acetate extract of *S. galbus* TP2 strain shows a potential antibacterial (MRSA, MSSA, MRCNS, VRE, *E. coli*), antifungal (*M. gypseum*), and anticancer (T47D cell line) activities. Many strains of *S. galbus* have been shown to be potential agents against fungal pathogens. A non-polyenic structure from *S. galbus* has been reported to be active against pathogenic fungi (Paul and Banerjee 1983). The metabolite from ethyl acetate extract prepared from the *S. galbus* novel strain with pesticidal activities against lepidopteran insects has been patented (Streptomyces galbus strain with insecticidal activity and method of using as an insecticide, European Patent EP1272611). The results described in this article indicate that this microorganism can be useful for many applications, such as control of infectious diseases and drug discovery (Sukandar *et al*. 1984).

Acknowledgments

The authors thank ITB, Ristek and LIPI for supporting the finance, facility and infrastructure.

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